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Preliminary X-ray crystallographic analysis of a novel maltogenic amylase from *Bacillus stearothermophilus* ET1

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Abstract

A novel maltogenic amylase from *Bacillus stearothermophilus* ET1, which has a dual activity of α-1,4- and α-1,6-glycosidic bond cleavages and α-1,6-glycosidic bond formation, was crystallized by using the hanging-drop vapor-diffusion method. The best crystals were obtained by employing a high concentration of protein (56 mg ml^{-1}) and a precipitant containing 22% glycerol, 1.6 *M* ammonium sulfate in 0.1 *M* Tris–HCl (pH 8.5). Native diffraction data to 2.66 Å resolution have been obtained from crystals flash-frozen at 110 K. The crystals belong to the space group $P2_12_12_1$ with unit-cell dimensions of a = 77.62, b = 121.23, c = 244.29 Å, and contain three or four protomers per asymmetric unit. Structure determination by multiple isomorphous replacement is in progress.

1. Introduction

Amylases are widespread enzymes in microorganisms, plants and animals. They hydrolyze starch materials, and are classified according to their enzymatic action. Glucoamylases (E.C. 3.2.1.3) and β-amylases (E.C. 3.2.1.2) are exo-type enzymes cleaving glucose and maltose units, respectively, from the non-reducing end of starch materials by hydrolyzing the α-1,4-glycosidic bonds. Pullulanases (E.C. 3.2.1.41) cleave the internal α-1,6-glycosidic bonds, while neopullulanase cleaves both the α-1,4- and the α-1,6-glycosidic linkages internally (Takashi *et al.*, 1991). α-Amylases (E.C. 3.2.1.1), on the other hand, catalyze the cleavage of the internal α-1,4 linkage of starch, glycogen and various oligosaccharides. Some of these are used in mass quantity as industrial enzymes (Forgaty, 1983; Thoma *et al.*, 1971). Although α-amylases exhibit varying action patterns depending on their sources, they share a number of common features: (1) a similar molecular weight of about 55 kDa; (2) the requirement of a calcium ion for enzyme activity and stability; (3) inhibition by acarbose, a pseudo-tetrasaccharide inhibitor of α-amylases and (4) similar three-dimensional structures despite limited sequence homology (Machius *et al.*, 1995). The crystal structures of different α-amylases (Brady *et al.*, 1991; Kadaziola *et al.*, 1994; Klein & Schulz, 1991; Larson *et al.*, 1994; Qian *et al.*, 1993; Takashi *et al.*, 1991) revealed that this family of proteins has a common folding motif, the $(\alpha/\beta)_8$-barrel for the first 2/3 of the polypeptide chain, and that the active site is located at the interface between one end of the barrel and the C-terminal domain (Farber & Petsko, 1990).

A novel amylase from *Bacillus stearothermophilus* ET1, a strain isolated from soil, was recently cloned and purified. This enzyme, which is hereafter referred as BSMA (*Bacillus stearothermophilus* maltogenic amylase), was found to exhibit unique characteristics that are different from other α-amylases (Cha, 1998). First, BSMA displays a dual activity of α-1,4- and α-1,6-bond cleavages. It hydrolyzes the α-1,4 linkages of β-cyclodextrin and pullulan as well as soluble starch. β-Cyclodextrin and starch are hydrolyzed mainly to maltose, and pullulan to panose (glucosyl-α-1,6-glucosyl-α-1,4-glucose) with a trace amount of maltose and glucose. Although neopullulanase hydrolyzes pulluan to panose efficiently (Hiroki *et al.*, 1992), its activity for starch hydrolysis, *i.e.* α-1,4-glycosidic bond cleavage is less efficient compared with that of BSMA, and it does not hydrolyze cyclodextrins. Second, BSMA is shown to have α-1,4- to α-1,6-transglycosylation activity. When 0.5%(*w*/*v*) solutions of malto-oligosaccharides of varying length were reacted with BSMA, they were hydrolyzed to maltose and glucose. However, when the concentration was increased to 5%(*w*/*v*), branched oligosaccharides were produced containing the α-1,6-linkage (Fig. 1). Third, BSMA hydrolyzes acarbose, a competitive inhibitor of α-amylases, to glucose and trisaccharide as is apparent on a thin-layer chromatogram of the hydrolysate (Cha, 1998). Until now, there has been no reported α-amylase with the activity of cleaving acarbose. Fourth, BSMA is inhibited by some divalent metal ions including the calcium ion which activates other α-amylases. The activity is decreased by about 20% in the presence of 5 m*M* $CaCl_2$ (Cha, 1998). Fifth, BSMA is composed of 590 residues with a calculated molecular weight of 69 627 Da, which is about 100 residues more than other α-amylases. Sequence alignment of 47 amylolytic and related enzymes revealed that the C-terminal region of BSMA (about 490 amino acids) exhibits high sequence homology with other α-amylases (Jespersen *et al.*, 1993). Thus, the 100 residues from the N-terminus of BSMA are likely to be responsible for its distinct catalytic properties.

Structures of several α-amylases (Larson *et al.*, 1994; Mizuno *et al.*, 1993; Tao *et al.*, 1989), β-amylases (Cheong *et al.*, 1995; Mikami *et al.*, 1994), glucoamylase (Aleshin *et al.*, 1992, 1996) and complexes of these enzymes with inhibitors (Aleshin *et al.*, 1994; Bompard-gilles *et al.*, 1996; Mikami *et al.*, 1993; Stoffer *et al.*, 1995) have provided valuable insights into their enzymatic mechanisms. However, there is no three-dimensional structure of any enzyme cleaving the α-1,6-glycosidic linkage (MacGregor, 1993). The three-dimensional structure of BSMA, therefore, will provide important information for understanding the novel characteristics mentioned above, especially the mechanism for the hydrolysis of the α-1,6-glycosidic linkage. It will also provide a structural basis for

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the activity of catalyzing the α-1,6-glycosidic bond formation at high oligosaccharide concentrations. Here we report the preliminary X-ray crystallographic analysis of BSMS crystals.

2. Results and discussion

2.1. *Enzyme purification*

The heterologous expression of the BSMA gene in *Escherichia coli* strain BL21 (DE3) was induced by 1 m*M* isopropyl-β-thiogalactopyranoside. Cell paste collected from a 6 l culture was resuspended in 20 m*M* Tris–HCl (pH 7.5) containing 2.5 μg ml^{-1} DNase I. After cell disruption by sonication, the crude cell lysate was incubated for 1 h at 333 K and then centrifuged for 1 h at 15 000 rev min^{-1} at 277 K. The heat treatment is based on a thermal inactivation experiment which showed that 10% of the BSMA activity was lost after incubation at 333 K for 84.6 min. Many other *E. coli* proteins were precipitated during the heat treatment. After centrifugation, the clear cell lysate was loaded successively onto Q–Sepharose and DEAE–Sephacel (Pharmacia Biotech, Sweden) columns equilibrated with the buffer containing 20 m*M* Tris–HCl (pH 7.5), and eluted with a linear NaCl gradient from 0.15 to 0.5 *M* in the same buffer. Further purification was achieved by a cation exchanger Hitrap SP column and a anion exchanger Mono-Q HR 5/5 column (Pharmacia Biotech, Sweden) using fast protein liquid chromatography (Waters, USA). The final concentrated enzyme solution was at least 95% pure as judged by SDS–PAGE.

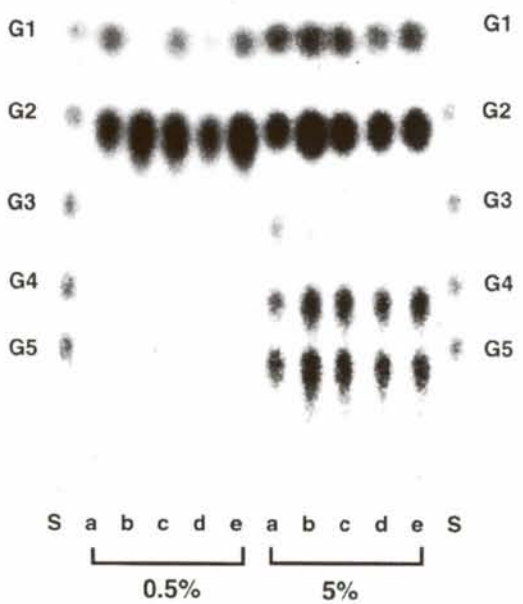

Fig. 1. TLC analysis of the transglycosylation activity of BSMA. Solutions containing 0.5 or 5%(*w*/*v*) malto-oligosaccharides (G3 to G7) were incubated with BSMA (400 CU g^{-1} substrate see below) at 328 K for 4 h. Lane *a* was spotted with the reactant of maltotriose solution (G3), lane *b* with maltotetraose (G4), lane *c* with maltopentaose (G5), lane *d* with maltohexaose (G6) and lane *e* with maltoheptaose (G7). G1–G5 were used as standards. The solutions containing 5%(*w*/*v*) malto-oligosaccharides produced branched oligosaccharides composed mainly of four or five glucose units (G4, G5) as the result of the α-1,6-glycosidic linkage formation by the enzyme. One CU (cyclodextrin unit) is defined as the amount of the enzyme which hydrolyzes β-cyclodextrin [0.5%(*w*/*v*)] in 30 min at 328 K at pH 6 (50 m*M* sodium citrate buffer) to produce reducing sugars equivalent to unit-absorbance change at 575 nm by the glucose–DNS assay (Miller, 1959).

2.2. *Protein crystallization*

Crystals of BSMA were obtained by the hanging-drop vapor-diffusion method using 24-well Linbro plates at 296 K. The first crystallization screening was performed with Crystal Screen^{TM}, the sparse-matrix screening kit from Hampton Research (USA). 2 μl of protein solution (10 mg ml^{-1}) in 20 m*M* Tris–HCl buffer (pH 7.5) was mixed with an equal volume of a precipitant solution on a cover slip, and the droplet of the mixture was allowed to equilibrate with 1 ml of the same precipitant. In the first screening, small thin plate-shaped multiple crystals appeared after 10 d in a mixture of the enzyme and the precipitant containing 1.5 *M* ammonium sulfate and 0.1 *M* Tris–HCl (pH 8.5), but the droplet containing crystals was turbid as a result of the protein aggregation. When we used 10% glycerol as an additive to the initial crystallization conditions, the aggregation problem disappeared, but it did not improve the size and quality of the crystals. It was found that increasing the protein concentration from 10 to 56 mg ml^{-1} resulted in larger but multiple crystals. The concentrations of glycerol and ammonium sulfate were systematically varied, and large single crystals (0.2 × 0.2 × 0.4 mm, Fig. 2) were obtained with a precipitant solution containing 22% glycerol, 1.6 *M* ammonium sulfate and 0.1 *M* Tris–HCl buffer (pH 8.5). For data collection, crystals were frozen at 110 K using a Cryostream cooler (Oxford Cryosystems, UK). It was not necessary to use any extra cryoprotectant to prevent the crystals from freezing damage. A 2.66 Å data set (89% completeness at 3.11–20 Å and 81% completeness at 2.66–20 Å, R_{sym} = 8.8%) was obtained using Cu $K\alpha$ radiation on a MAC Science DIP2020 imaging-plate system mounted on a M18XHF X-ray generator operated at 50 kV and 90 mA. Using an auto-indexing program provided with the program *DENZO* (Otwinowski, 1993) and examining the diffraction data set, we found that the crystals belong to the

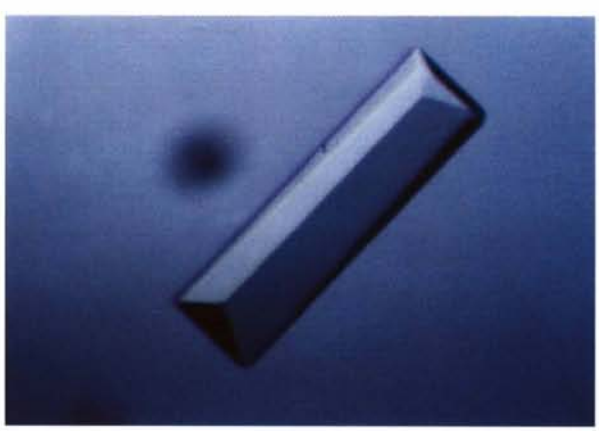

Fig. 2. A photograph of a BSMA crystal. The crystal size is 0.2 × 0.2 × 0.4 mm.

orthorhombic space group $P2_12_12_1$ with the unit-cell dimensions of a = 77.62, b = 121.23, c = 244.29 Å, and contain three or four molecules per asymmetric unit. This corresponds to the crystal volume per unit molecular weight (V_m) of 2.06 or 2.75 $Å^3$ Da^{-1}, respectively, given the molecular weight of BSMA (62 657 Da). Self-rotation searches have not provided a definite answer to the number of molecules in the asymmetric unit. The structure determination of BSMA by the multiple isomorphous replacement method is in progress.

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